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Studies on non enzymatic antioxidants of *Phyllanhus niruri L*

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ABSTRACT
Phyllanthus niruri L belongs to family Euphorbiaceae is a field weed. In the present investigation sequential solvent extracts of various plant parts and tissue isolates of *P. niruri* were analyzed for their non enzymatic antioxidant activity using various standard protocols such as DPPH free radical scavenging method and Ferric Reducing Antioxidant Power (FRAP). Results were expressed as antiradical efficiency (AE), which is 1000/ $IC_{50.}$

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Introduction

Oxygen derived free radicals have played a major role in the pathogenesis of a number of degenerative diseases. These free radicals molecules are released during the normal metabolic process of oxidation and thus can lead even to serious diseases like cancer, cardiac associated disorders (CAD), acceleration of the aging process and contribute to arthritis (Cross 1987). Natural foods and food-derived antioxidants such as vitamins, phenolics and phytochemicals have received growing attention because they are known to function as chemo-preventive agents against oxidative damages (Carrasco-Pancorbo *et al,* 2005; Perez-Bonilla *et al,* 2006; Valavanidis *et al,* 2004). Plants constitute an important source of active natural products that differ widely in terms of their structure and their biological properties. In recent years, the prevention of cancer and CAD has been associated with the intake of fresh fruits, vegetables, herbal teas that are rich in natural antioxidants (Virgili *et al,* 2001). The protective effects of plant products are due to the presence of several components that have distinct mechanisms of action; some are enzymes and proteins, while others are low molecular weight compounds such as vitamins, flavonoids, anthocyanins and phenolic compounds (Amro *et al,* 2002; Park *et al,* 2005).

Different antioxidant compounds act through different mechanism, no single method can fully evaluate the antioxidant activity. These assays have presented distinct challenges in evaluation of purified individual compound, mixed extracts, fractions, herbs, supplementation of vitamins and other chemicals/medicines. Thus Optimization of right method/s of application and evaluation is the need of time (Pellegrini *et al,* 1999)

Thus there are many different antioxidant components in plants and it is relatively difficult to measure each antioxidant component, separately. Owing to the complexity of the oxidation and anti-oxidation processes, no single testing method was found to be capable of providing a comprehensive picture of the antioxidant profile. Therefore, in the present investigation a multi-method approach was necessary to assess the antioxidant activity of biological samples (Koleva *et al,* 2002)

P. niruri commonly known as Stonebreaker because of its antilithic property. Various bioactivities such as antidiabetic (Okoli *et al,* 2011), anti-hepatotoxicity, (Ravikumar *et al,* 2011) antilithic, anti-hypertensive, antiplatelet aggregation activity (Kamal *et al,* 2012), antimicrobial activity (Mathur *et al,* 2012), anti-HIV and anti-hepatitis B (Bagalkotkar *et al,* 2011; Naik and Juvekar , 2003) have been reported.

Materials and Methods

Chemicals

Vitamin A, C and E, the stable free radical DPPH 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), TPTZ (2, 4, 6-tris (2pyridyl)-*s*-triazine) were obtained from Sigma chemicals (Sigma-Aldrich GmbH, Sternheim, Germany).

Tissue Culture

MS medium (Murashige and Skoog, 1962) was used for initiation and maintenance cultures of *P. niruri* . Nodal segments were surface sterilized with $HgCl_2$ solution, rinsed thrice with sterile distilled water; Explants were inoculated on Laminar flow hood fitted with ultraviolet light lamp, in the flasks containing culture medium aseptically. Cultured flasks were incubated in culture chamber with temperature maintained at 25 ± 1°C and 1200 lux light was provided for 16 h. The callus culture was established by using various treatment doses of hormones. These calli were maintained for about six months with frequent subculturings at time interval of 4-6 weeks. Growth index (GI) was calculated after 2, 4, 6 and 8 weeks of fresh sub culturing to record the growth pattern.

GI= Final wt of the tissue – initial wt of the tissue/ initial wt of the tissue

All the plant parts collected were cleaned and oven dried at 100ºC for 10 min to deactivate the enzymes and then at 25-28^{0}C till constant weight was achieved and then powdered. The voucher specimen of experimental plant was deposited in Herbarium of Department of Botany, University of Rajasthan, Jaipur (RUBL No. 30247).

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Screening and comparison of antioxidant activity of various plant parts and callus tissues were subjected to sequentially fractionated solvent extraction viz methanol fraction – MF; hexane fraction – HF; dichloromethane fraction – DCF and ethyl acetate fraction - EAF. All the fractions were subjected to determination of AE using established methods.

Determination of Non Enzymatic Antioxidant Activity

Different fractions were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH˙ (2, 2-diphenyl-1-picrylhydrazyl; Brand-Williams *et al,*. 1995). The percent of DPPH discoloration of the sample was calculated according to the formula

$$\textbf{Discoloration \%} = [1 - (Abs_{SAMPLE}/Abs_{CONTROL})] \times 100$$

The discoloration was plotted against the sample extract concentration and a logarithmic regression curve was established in order to calculate the IC_{50}. Ascorbic acid (vitamin C), vitamin A, E were used as a standard drug for comparing the activities of tested samples The results are expressed as antiradical efficiency (AE), which is 1000-fold inverse of the IC_{50} value (AE = $1000/IC_{50}$, Parejo *et al,* 2002).

FRAP Assay

The antioxidant power is counted in Trolox equivalents (Huang *et al,* 2005). The FRAP is another method of AE assay (Magalhaes *et al,*.2008). This assay is based on the reduction of Fe^{+++} to Fe^{++} due to the action of antioxidant activity. The Fe^{++} interacts with TPTZ providing a strong absorbance at 593 nm (Szydlowska *et al,* 2008; Varga *et al,* 1998).

Plant sample (1g) were cut into small pieces and mashed with a cool mortar and pestle using quartz sand and 9 mL cool 0.1M phosphate buffer was added. (pH 7.6, containing 0.1mM EDTA). Each of the test mixture was filtered through a filter paper and centrifuged at 15,000 rpm for 10 min. The supernatant was used for the measurements of OD (optical density) at 593nm after make up to 5mL volume.

Calculation: The relative activities of samples were assessed by comparing their activities standard curve of ferrous sulphate. Ferrous sulphate was dissolved in distill water and different concentrations (100-1000μM/L) was used for the measurement of OD. Standard FRAP reagent was used as blank.

Statistical analysis

Values are given as mean ± SEM (standard error of the mean) and were compared using one way ANOVA to judge the difference between various groups. Values of $p<0.05$ were considered statistically significant

The statistical error of mean was calculated by the following formula:-

$$S.E. = \frac{\sigma}{\sqrt{n}}$$

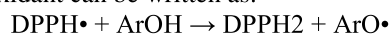
Where

σ = standard deviation

n = number of observations

The test of significance (t-test) was calculated by the following formula:

$$t = \frac{m_1 - m_2}{\sqrt{(SEM_1)^2 + (SEM_2)^2}}$$



Where,

m_1 = mean of one set of values.

m_2 = mean of second set of values.

SEM_1 = standard error of the first set of values.

SEM_2 = standard error of the second set of values.

The probability ‘p’ for obtaining ‘t’ value of at least as great as the calculated one for a given number for the degree of freedom was found in the Fisher’s table.

The p - values were signified according to the following conventions.

$P<0.05$ = difference was almost significant.

$P<0.01$ = difference was significant.

$P<0.001$ = difference was highly significant

Results

Tissue culture

Unorganised callus was established from nodal segments inoculated on MS medium supplemented with IBA at treatment dose of $2mgL^{-1}$, after trying various hormonal treatment doses. Growth index (GI) was calculated at various time intervals of 2, 4, 6 and 8 weeks of fresh subculturings. The GI was found to be minimum in 2 weeks (0.74) and maximum (2.34) in 6 weeks old tissue. Thereafter, the GI steadily declined thus showing a characteristic sigmoid curve.

DPPH Assay

The free radical (DPPH˙) scavenging activity expressed as AE ranged from 95 to 124. The free radical (DPPH) scavenging activity of *P. niruri was* found to be comparable to the well known antioxidants such as vitamin C (Table 1)**.**

FRAP Assay

AE ranged from 90.00 to 170.8. Highest activity was observed in MF of stem (170) and lowest in HF of stem. (90.00) The values were based on standard regression curve of ferrous sulphate and calculated as mM/l/g (Table 2).

Discussion

Unorganised callus was established from nodal segments inoculated on MS medium supplemented with BAP and IBA at treatment dose of $3mgL^{-1}$, each after trying various hormonal treatment doses. Growth index (GI) was calculated at various time intervals of 2, 4, 6 and 8 weeks of fresh subculturings. The GI was found to be minimum in 2 weeks (0.75) and maximum (3.06) in 6 weeks old tissue. Thereafter, the GI steadily declined thus showing a characteristic sigmoid curve. An increase in GI after supplementation of various growth regulators also finds support from the observations of biotechnologists that the growth of tissue sometime depends upon the culture medium and also controlled by the environmental and biological factors, like, pH, dose and combinations of growth regulators used (Barz and Ellis 1981).There is no uniform and clear definition of growth of plant cell cultures and dry weight or fresh weight methods have been in use for determining growth index (GI), because of its preciseness, accuracy in observing variation (Grossmann 1988). The maximum GI was achieved at the 6th week of subculture indicating the exponential growth phase. Minimum GI was observed at 2nd week of subculture.

Free radicals are reactive species with an unpaired electron. Antioxidants are able to reduce free radicals by donating an electron or hydrogen atom to the free radical. The hydrogen atom transfer (HAT) activity of plant extracts was studied using the DPPH• free radical and its reaction with a phenolic antioxidant can be written as:

DPPH• + ArOH → DPPH2 + ArO•

In the present report maximum AE was observed in herbal formulation which may be due to synergetic effect of various plant parts. Harish and Shivanandappa (2006) reported the hepatoprotective and antioxidant activity of *P. niruri*.

FRAP assay is based on the reduction of Fe^{+++} to Fe^{++} due to the action of antioxidants. Subsequently, the Fe^{++} formed may interact with TPTZ providing a strong absorbance at 593 nm (Szydlowska *et al,* 2008). In the present study various sequential extracts of various plant parts and callus tissue showed higher AE when compared with standard regression curve of ferrous sulphate indicated superior AE of *P. niruri* extracts.

There are many different antioxidant compounds in plants and it is relatively difficult to measure each antioxidant component separately, which may have supplementary or complementary effect in the given test extract leading to various antioxidant activities and actions. Therefore, multi method approach to determine the antioxidant efficacy is being preferred.

Conclusion

From the present study, it can be concluded that *P. niruri* is a good source of non-enzymatic antioxidant components. The antioxidant activity may be due to inhibiting the formation of radicals or scavenge the formed radical s and it may be due to the presence of the phenolic compounds. *P. niruri* could thereby provide a useful source of antioxidants in oxidative stress related disorders.

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Table 1

Antiradical Efficiency (AE) of *P. niruri* Plant Parts and Callus Cultures using DPPH Radical Scavenging Assay

Fractions	AE of Various Plant Parts and Callus				
	Leaves	Stem	Roots	Callus	AE of Reference Compound
MF	118.34±0.98	77.76 ±0.70	101.62 ±0.88	95.40±1.31	Vitamin C 176.36± 1.22

Each value is a mean of three replicates ± SE

Table 2
Antiradical Efficiency (AE) of *P. niruri* Plant Parts and Callus Cultures using FRAP Assay

Fractions	Various Plant Parts			
	Leaves	Stem	Roots	Callus
MF	126.4	170.8±0.57	140±1.5	122±1.455
HF	104 ±1.555	90 ±0.382	112 ±2.51	120±2.685
DCF	110±1.95	98 ±0.485	125 ±1.0	129 ±2.270
EAF	105 ±1.77	124 ±0.230	119 ±1.455	94 ±2.375

Where; MF – Methanol Fraction; HF – Hexane Fraction; DCF - Dichloromethane Fraction; EAF – Ethyl Acetate Fraction